Herpesvirus Infection Enhances Cholesterol and Cholesteryl Ester Accumulation in Cultured Arterial Smooth Muscle Cells

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In our previous experiments, atherosclerosis similar to that in humans was reproducibly induced in both normocholesterolemic and hypercholesterolemic specific-pathogen-free (SPF) chickens by infection with Marek's disease herpesvirus (MDV). In contrast, uninfected chickens fed either relatively cholesterol-poor or cholesterol-supplemented diets did not develop this arterial disease. In experiments reported here, the hypothesis that infection of arterial smooth muscle cells (SMCs) with MDV would enhance lipid accumulation in these cells was tested. The number of MDV-infected SMCs with lipid stained with oil red O was assessed, and the lipid content of these cells was quantitated chemically by chromatographic and fluorometric analyses. These data were compared to those of uninfected control cells and, in the case of chemical analyses, were also compared to SMCs infected with a second avian herpesvirus, turkey herpesvirus (HVT). Results indicate the following: 1) The percentage of MDV-infected SMCs containing stainable lipid was significantly greater than the percentage of uninfected SMCs; 2) Increased total lipid accumulation was observed in MDV-infected SMC, particularly cholesterol (CH) and cholesteryl esters (CEs), as compared with uninfected or HVT-infected cells; 3) The types of CEs and nonesterified fatty acids (NEFA) accumulating in MDV-infected cells (particularly saturated types of CEs and NEFAs) were significantly different than those in uninfected or HVT-infected SMCs. These qualitative and quantitative differences in lipid content between infected and uninfected SMCs suggest that infection with MDV results in altered intracellular lipid metabolism. Results support the hypothesis that lipid accumulation in arteries of normocholesterolemic chickens may result from MDV infection acting at the cellular level to induce lipid accumulation that resembles that in human atheroarteriosclerosis. (Am J Pathol 1981, 105:176–184)

RESULTS OF OUR PREVIOUS experiments indicate that atherosclerosis, closely resembling the chronic disease in humans, is induced in both normocholesterolemic and hypercholesterolemic chickens by infection with Marek's disease herpesvirus (MDV).[1,2] Uninfected chickens fed either cholesterol-poor or cholesterol-supplemented diets did not develop this arterial disease.[1,2] These results were reproducible in repeated experiments.[3] The pathogenesis of these arterial lesions is not well understood.[1-3] In particular, the mechanism of lipid accumulation in arterial lesions of MDV-infected normocholesterolemic chickens needs explication. Experiments reported here were designed to test the hypothesis that infection of cultured arterial smooth muscle cells (SMCs) with MDV would lead to lipid accumulation in these cells.

The hypothesis that herpesvirus infection of cells may lead to enhanced lipid accumulation was suggested by previous experiments of Fabricant et al.[4] Findings of these experiments indicated that infection

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with a feline herpesvirus of a variety of cultured cells derived from cats resulted in lipid accumulation. Results of experiments, reported here, indicate that infection of cultured chicken arterial SMCs with MDV will lead to the accumulation of lipids, particularly cholesterol (CH) and cholesteryl esters (CEs), in these cells. These *in vitro* findings suggest virus-induced alterations in the lipid metabolism of SMCs.

Materials and Methods

Cell Culture Medium

Culture medium used in these experiments consisted of equal parts of Leibowitz and McCoy's mediums (GIBCO, Grand Island, NY) supplemented with 20% fetal calf serum (LMF_{20} medium).[5] Ten mg of streptomycin and 100 units of penicillin were added per 100 ml of medium and the pH was adjusted to 7.4 with 8% sodium bicarbonate. Cells were cultured in Corning tissue culture (TC) flasks preconditioned in a 35 C incubator containing 3% CO_2 and 97% air.

Chicken Arterial Smooth Muscle Cells

Smooth muscle cells were derived from brachiocephalic arteries or proximal descending aortas of 7-week-old specific-pathogen-free (SPF) male P-line chickens, the same line of chickens used in our previous *in vivo* experiments.[1-3] Birds were killed by cervical dislocation and 1–3-cm segments were obtained aseptically from the brachiocephalic arteries or proximal aortas.

Arterial segments were washed several times with phosphate-buffered saline (PBS, pH 7.4) to remove traces of grossly visible blood. The segments were then slit longitudinally, and the adventitial layer was stripped and discarded. Care was taken to avoid injury to the remaining intimal–medial layer. The intima–media was minced into pieces approximately 2 sq mm in size and washed twice with PBS. Five to ten pieces of the arterial segment were transferred to 25-sq cm TC flasks containing 4 ml of LMF_{20} medium (the quantity of medium was adjusted to avoid floating the tissues), and the flasks were incubated at 35 C in 3% CO_2.

Culture medium (LMF_{20}) was replaced at 48-hour intervals. When an estimated 30% of the cell monolayer of primary SMC had explanted (6–7 days), the cells and tissue pieces were trypsinized for approximately 2 minutes with 0.5% trypsin-EDTA (wt/vol) in PBS, pH 7.5. (This treatment of tissues significantly reduced the time required for explantation of primary cells). The length of time for trypsinization was carefully monitored and depended on trypsin lot. Explanted cells were suspended in 2 ml of LMF_{20} and transferred to a second 25-sq cm TC flask. The remaining tissue pieces were rinsed twice with 1.5 ml of LMF_{20} each time to remove residual primary cells. These rinses were combined with the cell suspension in the second flask. The tissue pieces were resuspended in approximately 4 ml of LMF_{20} and transferred to a third 25-sq cm TC flask or left in the original flask. Flasks with transferred cells and tissue pieces were incubated, as previously described, with medium replaced at 48-hour intervals. It is noteworthy that primary SMCs could continue to be explanted from arterial tissue for periods of 25–40 days, providing there was no evidence of tissue necrosis. Cultured cells were subpassaged when monolayers developed in 3–6 days. (Cultures 7 or more days old were found unsuitable for use because they were overgrown, and the cells neither trypsinized uniformly nor maintained well in subpassage.) Optimal subcultures were obtained when resulting monolayer cell suspensions were split 1:3 and seeded at concentrations of approximately 1–2 $\times$ 10^5 cells/ml of medium.

All cultured cells were examined by phase-contrast microscopy prior to their use in experiments and were also routinely examined by electron microscopy to assure their identity as SMCs. Cells grew in a pattern characteristic of cultured arterial SMCs[6]. Only SMC cultures with uniform spindle-shaped, agranular cells that had centrally located nuclei, minimal vesicle formation on cell membranes, and no cytoplasmic vacuoles were used for the experiments. In addition, only cells representing the third subpassage (rarely the fourth) were used.

Freezing Arterial Tissue and SMC

Pieces of intimal–medial arterial tissue and SMC were frozen in liquid nitrogen (LN_2) for future retrieval. Cell culture medium (LMF_{20}) with 5% dimethylsulfoxide (DMSO), cooled at 4 C, was used as the freezing medium. Two to four tissue pieces, each approximately 10 $\times$ 4 mm, were frozen in 5-ml cryules with 2–3 ml of medium. Cells from cultures 3–5 days old were used for freezing. Approximately 5–10 $\times$ 10^5 cells were suspended in 1 ml of the freezing medium in 1-ml cryules. The cryules were flame sealed and placed at -70 C for 30 minutes, after which they were stored in LN_2 until needed.

When retrieved for culture, the cryules were thawed rapidly in a covered container with cold water (a few drops of Clorox were added to the waterbath

to avoid a possible contamination of cells or tissue). Tissue pieces were washed once with PBS and once with LMF_{20}, after which they were minced and cultured as described for unfrozen tissue. Cells were placed in 25-sq cm flasks and cultured as described above, with the exception that growth medium was replaced at 24 hours (to remove traces of DMSO) and at 48-hour intervals thereafter.

Electron Microscopy

Smooth muscle cells for transmission electron-microscopic (TEM) examination were grown in 25-sq cm TC flasks. Confluent cell monolayers were washed four or five times with serum-free culture medium and fixed in Karnovsky's fixative diluted 1:1 with serum-free culture medium for 30 minutes at room temperature. The cells were then washed four or five times at 10-minute intervals with 0.1 M cacodylate buffer, postfixed for 30 minutes at room temperature in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.3, washed with 0.1 M cacodylate buffer, and dehydrated in increasing concentrations of ethyl alcohol. They were then embedded in Epon 812 by the method of Ross.[6]

Viruses and Uninfected Chicken Skin Extract Used for SMC Inoculations

The CU-2 clone-purified strain of MDV used in our previous experiments was used to inoculate cultured SMCs for these experiments.[1-3] Turkey herpesvirus (HVT), a virus which has been used as a vaccine to prevent MDV-induced tumor formation in chickens,[7,8] was also used in these investigations to test the specificity of the MDV effect on SMC. The HVT used was the clone-purified 752 strain of virus (Merck FC 126 vaccine strain).

Both MDV and HVT replicate in chicken and turkey feather follicle epithelium, respectively. Therefore, MDV- and HVT-cell-free virus stocks are prepared from extracts of skin with feather follicle epithelium known to contain virus.[8] The chicken "skin extract" for these experiments was prepared from skin of SPF uninfected P-line chickens in a similar manner to that used for the preparation of cell-free virus stocks. Smooth muscle cells were inoculated with a quantity of uninfected skin extract equivalent to that contained in the virus inocula to control for the possible effects of the skin extract.

Inoculation of SMCs

Cell cultures for lipid studies were trypsinized as described above and suspended in LMF_{20} medium for subsequent division into three equal aliquots. Cell suspension containing $1-2 \times 10^5$ cells/ml of medium were placed in sterile flasks in an ice bath and stirred for 2 minutes on a magnetic stirrer. This suspension was divided into three 150-sq cm flasks. One flask remained uninoculated, a second flask was inoculated with uninfected skin extract (diluted as the virus stock), and the third flask was inoculated with five plaque-forming units (PFU) of either MDV or HVT per ml of cell suspension. All flasks, inoculated or uninoculated, were placed in an ice bath and stirred on a magnetic stirrer for 2 additional minutes to disperse the inocula and enhance infection of cells with virus. Sets of eight Leighton tubes containing coverslips were seeded with 1 ml/tube of the appropriate cell suspension (uninoculated, inoculated with uninfected skin extract, or inoculated with either MDV or HVT). The total volume of cell suspension remaining in each 150-sq cm flask was 30 ml. Following inoculation, all cultures were incubated at 35 C in 3% CO_2 as described above. Culture medium was replaced only after 24 hours, and the cultures were incubated for a total of 6 days.

Staining

Four Leighton tube coverslips with SMC culture monolayers from each infected or uninfected culture were stained with oil red O and counterstained with hematoxylin. Optimal results were obtained for the oil red O stains after fixation of SMC by immersion of coverslips in 10% buffered formalin containing a few drops of ammonia water. Four parallel coverslips of SMC cultures were also stained with May-Grünwald-Giemsa stain.

Counting Cells Stained with Oil Red O

The percentage of cells containing oil-red-O-positive lipid was determined by counting 500 SMCs at 625× in approximately 15 randomly chosen fields. Smooth muscle cells were counted in areas where they grew as a monolayer and were counted as positive regardless of the amount of lipid staining in each cell. Coverslips were evaluated without knowledge of treatment group.

Harvesting Of SMC Cultures For Lipid Analyses

After 6 days of incubation, the supernatant fluids (postculture medium) were decanted, frozen at −70 C, and stored for lipid analysis. The SMCs were harvested by being scraped off with a rubber

policeman and then suspended in 5 ml of cold Leibowitz-McCoy medium without serum in sterile centrifuge tubes placed in an ice bath. The TC flasks were washed three times with 2.0 ml of the same medium each time, and the washes were added to the cell suspension. The cells were pelleted at 1000 rpm in an International Centrifuge for 15 minutes and the supernatant was discarded. The pelleted cells were resuspended in cold sterile PBS and repelleted as above. The supernatant was replaced with 1 ml of cold sterile PBS containing 1% DMSO without disturbing the pellet. Cell pellets under N_2 (g) were placed at −20 C until the supernatant liquid was frozen, after which they were stored at −70 C until quantitative lipid analyses could be performed (within 2–3 weeks). Preliminary studies revealed that freezing of infected or uninfected SMC and postculture fluids under these conditions did not alter their lipid content.

Lipid Analysis

All glassware used for the lipid analyses was washed with chromic acid cleaning solution and rinsed thoroughly with double distilled water. Only highly purified, distilled organic solvents were used. The pelleted infected and uninfected SMCs for lipid analyses were washed twice with PBS (pH 7.2) and repelleted after each wash as described above. Pellets were resuspended in 1.5 ml of PBS at 4 C (pH 7.2) and homogenized continuously for 3 minutes with a Duall homogenizer in an ice bath. Analysis of DNA was done on a 0.5-ml aliquot of the homogenate by the Kissane and Robins microfluorometric method with 3,5-diaminobenzoic acid[9] as modified by Hinegardner.[10] Lipids were extracted from the remaining 1.0-ml aliquot of cell homogenate by the method of Folch et al.[11] The Folch extract subsequently was dissolved in 10 ml of dichloromethane–methanol (2:1 vol/vol).

Five milliliters of the dichloromethane–methanol solution were used for total lipid analysis. Since unsaturated bonds of various lipids have been shown to interfere with fluorometric quantitation,[12] lipid analysis was performed after exhaustive hydrogenation of extracted lipids with platinum oxide as the catalyst. Following hydrogenation, which was routinely confirmed by gas–liquid chromatography, the lipid extract was filtered through fat-free Whatman No. 4 filter paper, evaporated to dryness under N_2 (g) and redissolved in 10.0 ml of dichloromethane–methanol (2:1 vol/vol). A 4.0-ml aliquot and various concentrations of a standard lipid mixture[13] were fractionated by thin-layer chromatography (TLC) on silica gel chromatoplates (Whatman K5, 80Å, 250 μ thick) into six major lipid classes: phospholipids, cholesterol, nonesterified fatty acids, triacylglycerols, cholesteryl esters, and squalene (hydrocarbons).[13] The various lipid classes were identified by spraying the TLC plate twice to saturation with 0.001% Rhodamine 6G. All lipid classes except phospholipids were quantitated *in situ* with a Turner Model 111 fluorometer.[13] Phospholipids that remain at the origin were scraped from the plate prior to identification of the lipid classes with Rhodamine 6G. They were eluted from the silica gel with chloroform–methanol (1:2 vol/vol) and quantitated colorimetrically with 0.02% malachite green according to the method of Chalvardjian and Rudnicki.[14]

The remaining 5.0 ml of unhydrogenated dichloromethane–methanol lipid solution of the Folch extract was used to profile and quantitate nonesterified fatty acids (NEFAs) and fatty acids esterified to cholesterol (CEs) by gas–liquid chromatography (GLC). For quantitation by GLC, 0.1 ml of 10 mg/ml heptadecanoic acid solution dissolved in dichloromethane (Applied Science Laboratories, State College, Pa) was added to each sample as an internal standard prior to separation of NEFAs and CEs from other lipids by TLC. The lipids were then separated by TLC as described above. Nonesterified fatty acids and CEs were identified by the use of 0.001% Rhodamine 6G, and they were eluted separately from the silica gel chromatoplates with diethyl ether by the method of Goldbrick and Hirsch.[15] The diethyl ether was removed by evaporation under a stream of N_2 (g) at 30 C. Five ml of methanol–dichloromethane (6:1 vol/vol) were added to the lipid residue for direct micromethanolysis with boron trichloride gas to prepare fatty acid methyl esters.[16] Qualitative and quantitative GLC analyses were performed by established procedures with a polar polyester support column consisting of 10% Supelcoport-222 PS, 100/120 mesh (Supelco Inc., Bellefonte, Pa) and a Hewlett Packard 7620A chromatograph with a model 3375 integrator. The column was calibrated with Applied Science Fatty Acid K-108 standard mixture, which agreed with the stated composition data with a relative error of less than 4% for major components. Standard fatty acid methyl esters showed a linear response over the range of sample sizes analyzed, and gas chromatographic peaks were identified by comparing retention times with reference standards.

Statistical Analysis

The mean concentrations of each lipid class iso-

lated from uninfected controls, MDV-infected cells, and HVT-infected cells were compared by the Kruskal-Wallis analysis of variance by ranks.[17] Lipid data derived from the postculture medium and the mean concentrations of the different NEFAs and CEs were treated statistically in a similar manner. Subsequent pairwise comparisons between the three groups of cells were performed by nonparametric multiple comparisons.[17]

Results

Cell Culture Observations

Arterial explants obtained from brachiocephalic arteries of 7-week-old chickens consistently produced better SMCs than explants obtained from proximal aortas or from the arterial tissue of older chickens. Smooth muscle cells grew optimally when cultured at densities greater than 1×10^4 cells/ml in LMF_{20} and incubated at 35 C as compared with cells grown in mediums containing other serums (horse serum or homologous or autologous chicken serum) or at higher temperatures (36–41 C, temperatures frequently found to be optimal for other avian cells). Cells explanted from arteries had the typical growth pattern and microscopic appearance of SMCs.[6] These cells developed a growth pattern resembling "hills and valleys" if incubation was continued after monolayer formation. By light-microscopic examination, they were spindle-shaped cells with centrally located oval nuclei and prominent nucleoli. Marked cellular pleomorphism, as indicated by changes in size, shape, and syncytial formation, and pronounced vesicle formation, was frequently noted in the SMCs of the sixth passage, perhaps indicating a change from the diploid to heteroploid phase. Therefore, SMCs used in experiments were in the third, rarely the fourth, cell passage. By electron-microscopic examination, cells were homogeneous and contained variable quantities of cytoplasmic filaments and dense bodies and relatively few organelles, including mitochondria, rough endoplasmic reticulum, and free ribosomes.[6] Thus, both with regard to their growth characteristics and ultrastructural features, these cells had characteristics of cultured smooth muscle cells described by others. Fibroblastlike cells were not seen.

Infection of SMCs with MDV or HVT was evidenced by cytopathogenic effects, characterized by nuclear changes, syncytial cells, cytomegalic cells, and rounded cells. These changes were not observed in the uninoculated SMCs or SMCs inoculated with skin extract.

Histochemical Observations

There was a significantly greater percentage of MDV-infected SMCs containing oil-red-O–positive lipid than uninfected SMC (Table 1). Results from three separate experiments indicated that 43–61% of SMCs infected with MDV contained oil-red-O–staining lipid in cultures infected with 10^{-5}–10^{-2} dilutions of MDV. This was significantly greater than the number of stained cells in the uninoculated cultures (11–18%) and cultures inoculated with uninfected chicken extract (16–23%) ($P < 0.05$). Although the amount of stained lipid in individual cells was not quantitated, it appeared that MDV-infected SMCs had more oil-red-O–positive material than did uninfected cells. In addition, crystals were observed in the cytoplasm of some MDV-infected SMCs but not in uninfected cells. These crystals had characteristics of known standard CH crystals when examined with a polarized light microscope.

Lipid Quantitation

Results of chemical analyses indicated that lipids in MDV-infected cells were quantitatively and qualitatively different from those in uninfected or HVT-infected cells. The total lipid in cultures of MDV-infected SMCs was significantly greater than in the uninfected controls (Table 2). This increase in lipid was due to a significant increase of CH, CEs, NEFAs, squalene, and phospholipids. Particularly striking was a seven fold increase in CEs and a four- to five-fold increase in CH and phospholipid. The total lipid content of SMC cultures infected with MDV was also significantly greater than the content of SMCs infected with HVT. The amounts of CH, CEs, phospholipids, and squalene were all significantly greater in SMCs infected with MDV as compared with cells infected with HVT. Of the various lipid classes measured, only CH was greater in HVT-infected than in uninfected controls. There were no significant differences in the total lipid content or in the individual lipid classes between the uninoculated SMCs and SMCs inoculated with uninfected skin extract in ten separate replicate analyses (Table 2).

The most striking quantitative difference between MDV-infected SMCs and uninfected SMCs or SMCs infected with HVT was the increase in CEs (Table 2). In addition to these quantitative differences, there were also qualitative differences in the CEs of SMCs infected with MDV when compared with either uninfected SMCs or SMCs infected with HVT (Table 3). In MDV-infected SMCs, there was an increase in CE-containing saturated fatty acids, ie, myristic, palmit-

Table 1 – Percentage of Uninfected and MDV-Infected Smooth Muscle Cells (SMCs) Stained With Oil Red O

Experiment	Number of culture replicates	Dilution of MDV or uninfected skin extract inoculum	Percentage of Oil Red O Stained SMCS		
			Uninoculated	Inoculated with uninfected skin extract	Inoculated with MDV
1	2	10^{-2}*	11 ± 1†	16 ± 1	61 ± 1
2	8	10^{-3}	15 ± 3	ND	52 ± 10
3‡	4	10^{-2}	18 ± 5	23 ± 3	54 ± 5
	4	10^{-3}		ND	58 ± 3
	4	10^{-4}		ND	45 ± 6
	4	10^{-5}		ND	43 ± 5

* Dilution corresponds to 5 plaque-forming units (PFU) of MDV/ml of cells. Suspension of 1-2 × 10^5 cells/ml.
† Mean ± SEM
‡ Experiment 3 used 4 different dilutions of the virus.
ND = not done.

ic, and stearic acids. There was also a decrease in the polyunsaturated fatty acids esterified to CH in cells infected with MDV or HVT as compared with uninfected controls.

The quantity of NEFAs was also significantly increased in SMCs infected with MDV when compared with uninfected controls (Table 2). As was the case with CEs, there were also qualitative differences in NEFAs of MDV-infected SMCs when compared with HVT-infected cells or uninfected cells (Table 4). These differences paralleled those seen for CEs and were in large part accounted for by an increase in saturated fatty acids, ie, palmitic and stearic acids. In the HVT-infected cells, there was significantly more polyunsaturated NEFAs (linolenic and arachidonic acids) than in the uninfected cells or the MDV-infected cells.

Analyses of the medium used to culture all four groups of SMCs revealed the following results (based on 9 separate replicates in μg/ml): CH: 0.17 ± 0.05; CE: 0.33 ± 0.10; NEFA: 0.27 ± 0.06; triacylglycerols (TG): 0.33 ± 0.11; phospholipids: 0.13 ± 0.04; and total lipid: 1.23 ± 0.07. In other studies, the postculture mediums from the infected and uninfected cultured cells were also analyzed for total lipid content. Results of five replicates analyzed revealed that the content of postculture mediums from MDV-infected and HVT-infected cells contained 10.0 ± 0.7 μg/ml and 13.7 ± 0.9 μg/ml total lipid, respectively, as compared with uninfected cells, which contained 6.6 ± 0.3 μg/ml total lipid. With respect to individual lipid classes, TG in the postculture media from MDV-infected SMC (1.70 ± 0.60 μg/ml) and HVT-infected SMC (1.60 ± 0.40 μg/ml) as well as p-lipid (MDV-infected SMC, 3.40 ± 1.20 μg/ml; HVT-infected SMC, 1.90 ± 0.70 μg/ml) were significantly greater ($p < 0.05$) as compared to the TG (0.33 ± 0.11 μg/ml) and p-lipid (0.13 ± 0.04 μg/ml) found in postculture media of uninfected cells.

Discussion

As demonstrated by the following observations, MDV-infected SMCs accumulated significant quantities of lipid when compared with either HVT-infected or uninfected SMC cultures: 1) Histochemical data revealed that significantly more MDV-infected SMCs than uninfected SMCs contained lipid, as demonstrated by oil red O stain. In addition,

Table 2—Total Lipid Content in Infected and Uninfected Arterial Smooth Muscle Cells

Lipid Class	Uninfected controls		Infected cells	
	Uninoculated (n = 10)	Skin extract (n = 10)	HVT-infected (n = 10)	MDV-infected (n = 10)
Cholesterol	1.36 ± 0.36^{a}*	1.00 ± 0.29^{b}	2.78 ± 0.23^{a,b}	4.81 ± 0.87^{a,b}
Cholesteryl esters	0.82 ± 0.26^{c}	0.73 ± 0.15^{d}	0.64 ± 0.10^{e}	4.94 ± 1.08^{c,d,e}
Nonesterified fatty acids	1.67 ± 0.20^{f}	1.39 ± 0.27^{g}	2.49 ± 0.45	3.20 ± 0.42^{f,g}
Triacylglycerols	0.70 ± 0.19	1.00 ± 0.18	1.92 ± 0.35	2.74 ± 0.97
Phospholipids	0.35 ± 0.04^{h}	0.44 ± 0.08^{i}	0.93 ± 0.20^{j}	1.97 ± 0.41^{h,i,j}
Squalene (hydrocarbons)	0.87 ± 0.13^{k}	1.22 ± 0.21^{l}	1.96 ± 0.37^{m}	3.57 ± 1.04^{k,l,m}
Total	5.72 ± 0.47^{n}	5.78 ± 0.59^{o}	10.72 ± 1.72^{p}	21.24 ± 2.95^{n,o,p}

* μg lipid/μg DNA ± SEM. Values with the same corresponding letters are significantly different ($P < 0.05$).

Table 3—Cholesteryl Esters Present in Infected and Uninfected Smooth Muscle Cells

Cholesteryl Esters	Uninfected Controls (n = 8)	HVT-Infected (n = 8)	MDV-Infected (n = 8)
Myristic (14:0)	0.07 ± 0.01[a]*	0.08 ± 0.01[b]	0.59 ± 0.03[a,b]
Myristoleic (14:1)	0.21 ± 0.02[c]	0.07 ± 0.02[c]	0.46 ± 0.03[c]
Palmitic (16:0)	0.23 ± 0.03[d]	0.24 ± 0.05[e]	1.76 ± 0.17[d,e]
Stearic (18:0)	0.18 ± 0.03[f]	0.11 ± 0.02[g]	0.80 ± 0.06[f,g]
Oleic (18:1)	0.27 ± 0.05	0.20 ± 0.02	0.13 ± 0.02
Linoleic (18:2)	0.20 ± 0.04[h]	0.02 ± 0.01[h]	Trace
Linolenic (18:3)	0.10 ± 0.02	Trace	Trace
Arachidonic (20:4)	0.11 ± 0.02	Trace	Trace

* μg/μg DNA ± SEM. Values with the same corresponding letters are significantly different ($P < 0.05$).

cholesterol crystals (as identified by polarized light microscopy) were found only in MDV-infected SMCs. 2) Quantitative chemical analyses revealed that CH, CEs, phospholipids, and squalene were significantly increased in MDV-infected SMCs as compared with either HVT-infected or uninfected SMCs. 3) Increases in saturated CEs consisting of myristate, palmitate, and stearate primarily accounted for the CE increase in MDV-infected cells as compared with HVT-infected or uninfected SMC. 4) Increased accumulation of NEFAs in MDV-infected SMCs was characterized by increases in saturated palmitic and stearic fatty acids relative to either HVT-infected or uninfected SMC controls. It may be concluded that the lipid alterations observed in MDV-infected SMCs are attributable to the specific effects of MDV infection on these cells. This may explain the significant lipid accumulation found in arterial lesions of MDV-infected normocholesterolemic chickens.[1-3]

The media of avian aortas contains two types of cells—typical vascular SMCs and an intralamellar connective tissue cell or intermediate cell of uncertain histogenesis that has characteristics of both fibroblasts and SMCs.[18] One may reasonably question whether or not there are two types of cells growing in these cultures derived from intima-media preparations of chicken aortas and which cell type is accumulating lipid. In all instances, cells grown from explants in our experiments were homogeneous and had the growth characteristics of SMCs. Furthermore, their ultrastructural features were like those of cultured vascular SMCs of other species that we have seen or others have described. We were not able to distinguish a separate population of fibroblastlike cells in our cultures. In this regard, our experience is similar to that of other investigators who have cultured cells from intima-media preparations of pigeon aortas.[19,20] Therefore, it appears either that intralamellar connective tissue cells do not grow out of the explants of avian arteries or that these cells are indistinguishable in culture from SMCs. Since the cells in our cultures were homogeneous and had the growth characteristics and ultrastructural features of SMCs, we have referred to them as such.

Table 4—Nonesterified Fatty Acids Present in Infected and Uninfected Smooth Muscle Cells

Nonesterified fatty acids	Uninfected controls (n = 8)	HVT-infected (n = 8)	MDV-infected (n = 8)
Myristic (14:0)	0.06 ± 0.01[a,b]*	0.21 ± 0.04[a]	0.14 ± 0.03[b]
Myristoleic (14:1)	0.04 ± 0.01[c,d]	0.42 ± 0.10[c]	0.26 ± 0.05[d]
Palmitic (16:0)	0.67 ± 0.11[e]	0.69 ± 0.06[f]	1.30 ± 0.09[e,f]
Stearic (18:0)	0.32 ± 0.06[g]	0.28 ± 0.05[h]	0.98 ± 0.09[g,h]
Oleic (18:1)	0.37 ± 0.07[i,j]	0.68 ± 0.10[i]	0.75 ± 0.10[j]
Linoleic (18:2)	0.33 ± 0.04	0.50 ± 0.05	0.34 ± 0.04
Linolenic (18:3)	0.08 ± 0.02[k]	0.17 ± 0.03[k,l]	0.04 ± 0.01[l]
Arachidonic (20:4)	0.07 ± 0.10[m]	0.15 ± 0.04[m]	0.03 ± 0.01[m]

* μg/μg DNA ± SEM. Values with the same corresponding letters are significantly different ($P < 0.05$).

The discovery that infection of cells with a virus can lead to significant lipid accumulation and perhaps alter lipid metabolism is consistent with our previous report.[4] Fabricant et al reported that feline cell cultures infected with a feline herpesvirus accumulated significant intracellular and extracellular lipid including CH crystals.[4] The crystals were identified as CH by structural analysis, polarized light-microscopic examination, and comparative mass spectroscopy with a known CH standard. These findings led the investigators to hypothesize a possible role for a herpesvirus in the pathogenesis of human atherosclerosis.[4,21]

Results of other investigators also suggest that infection with viruses can lead to intracellular lipid accumulation and altered lipid metabolism. For example, viruses that contain lipids (lipid envelopes) have been reported to alter lipids of cellular membranes.[22] Herpesviruses and adenoviruses may induce formation of specific lipoproteins, which may cause cell damage.[23] In addition, herpes simplex Types I and II viruses,[24] poxviruses,[25] Newcastle disease virus,[26] fowl plague virus,[26] and Semliki Forest virus[26] have been reported to alter the metabolism of lipids, ie, phospholipids, glycolipids, sphingolipids, and CH, in various infected cells. Furthermore, Blair and Brennan[27] reported increased phospholipid synthesis in chick fibroblasts infected with Sendai virus, and Mark-Malchoff et al reported increased threonine phospholipids[28] and marked accumulations of CEs, due in part to an increase in CH esterification and a decrease in CE hydrolysis[29] in polyoma-virus-transformed cells.

Mechanisms that may be involved in alterations

of intracellular lipid accumulation induced by virus infection include the following: 1) Virus-altered cellular surface characteristics may induce increased uptake of low density lipoproteins (LDL) via a LDL-receptor-mediated pathway or by bulk-phase endocytosis that transports CH and CEs into cells: 2) Infection may increase synthesis of CH and CEs from acetate due to the increased activities of β-hydroxy-β-methylglutaryl CoA reductase (HMG-CoA reductase) and fatty acyl CoA: cholesterol acyltransferase (ACAT), respectively; 3) Virus infection may induce changes in lysosomal and/or cytosolic cholesteryl esterase activities, thereby leading to variable pool sizes of CH and CE in cells. 4) Virus infection may induce changes in the rate of synthesis and/or oxidation of specific fatty acids, resulting in the accumulation of a greater proportion of saturated NEFAs. This mechanism may include modifications in the rate of fatty acid desaturation by the cytochrome b_5 oxygenase system. Since saturated NEFAs are particularly toxic to cells,[30] this effect suggests a reduced ability of these cells to oxidatively catabolize or excrete lipid.

Alterations in lipid metabolism resulting from changes in cellular enzyme activity induced by virus infection appear to be a likely mechanism to account for the lipid accumulation in the MDV-infected SMCs. However, the accumulations of saturated esters of CE argues against this possibility, since increased synthesis would be expected to result in accumulation of cholesteryl oleate.[31] Accumulation of saturated esters of CH suggests the possibility that the accretion of CEs results from increased uptake of lipoprotein. This also appears unlikely since the lipid content of the LMF_{20} was minimal (see Results). It may be that changes in lipid metabolism induced by infection with MDV are not comparable to those previously reported.

At this time, we cannot state which mechanisms cause the lipid accumulation in MDV-infected SMCs as compared with either HVT-infected or uninfected SMCs. However, whatever mechanisms are involved, the increased lipid accumulation, particularly of CH and saturated CEs and NEFAs, in MDV-infected cultured SMCs may be very important in the pathogenesis of MDV-induced atherosclerosis. Recent preliminary *in vivo* observations of Hajjar et al[32] are consistent with the *in vitro* findings reported here. The *in vivo* experiments indicate that normocholesterolemic chickens infected with MDV also have significantly greater accumulations of CH, CEs, and NEFAs in the aorta than do uninfected chickens. It is also noteworthy that in the studies reported here and the preliminary results of *in vivo* chemical analyses, the increased lipid in MDV-infected SMC cultures and aortas of infected birds was characterized particularly by increased CH and CEs—the two principal lipids accumulating in human atherosclerotic arteries.[33-35]

In conclusion, the results of our *in vitro* experiments support the hypothesis that MDV infection acts at the cellular level to induce specific lipid accumulation in arterial SMCs. Results suggest that this lipid accretion is a consequence of altered lipid metabolism induced by MDV. Finally, the findings suggest that MDV-induced alterations of cellular lipid accumulation may be of major significance in the pathogenesis of MDV-induced atherosclerosis.

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